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SUBSTANTIVE FUNGICIDES*

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ABSTRACT

Most leather fungicides are fat-soluble, and hence tend to be located in the grease and gradually lost as this migrates to the surface. The possibility that a compound having a fungicidal group could be bound to the protein, thus giving complete substantivity, is an attractive one, but stereochemical and other considerations suggest that its effectiveness would be very limited.

Probably more effective would be a compound which could be bound to collagen and which also possessed a fungicidal group that could be split off, preferably under the warm moist conditions favoring mold growth.

It has been shown that conventional fungicides, such as *p*-nitrophenol (PNP), 2-mercaptobenzothiazole (MBT), and *o*-chloroaniline (OCA), can be bound to collagen, using cyanuric chloride as an intermediary. The rate of release of free fungicide was determined under various conditions of pH and humidity. PNP is released slowly under normal conditions of storage and more rapidly under conditions conducive to mold growth. Indications are that protection will be maintained over periods of one to three years, depending on the temperature and humidity and the rate at which the free PNP is leached out. MBT is released more slowly and may, therefore, be more suitable in some circumstances. There is no evidence that OCA, bound through its amino group, is released at all, even at high humidities.

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INTRODUCTION

The growth of mold on leather can be a problem under warm, moist conditions and the incorporation of a fungicide is often considered desirable, especially in leathers to be used in the tropics. A fungicide for leather must have a general

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all-round toxicity and be effective against a wide variety of mold species growing on a number of different substrates, *i.e.,* the skin protein itself, grease, carbohydrate, and vegetable tan or other tanning materials. Most fungicides at present in use are fat-soluble, and hence tend to be located in the grease and gradually to be lost as this migrates to the surface.

The possibility that compounds having a fungicidal action could be bound to the protein and so have complete substantivity is an attractive one. Such a compound would have to have a group capable of reacting with the protein or with a suitable intermediary, and also an atom or group having fungitoxic properties. Whether a potentially biocidal group can still be effective when its mobility is restricted by attachment to the protein is an interesting question, on which little direct information appears to be available (see Hueck-van der Plas (1)).

The site of toxic action by the biocide may be inside the fungal cell, at the cell membrane, or even outside the cell, but it is thought probable that most fungicides, to be fully effective, must penetrate within the cell. Provided the bound fungitoxic group is able to come into close enough contact with the cell to do this, there appears to be no evidence to suggest that covalent binding limits its effectiveness. Clearly, loss of mobility must greatly reduce the chances of close contact between the spore and the fungicidal group, and consideration of the steric factors involved shows that, while it is conceivable that a bound fungicide can be effective as far as the substrate to which it is bound is concerned, its range will be severely limited and is unlikely to extend through the vegetable tan, grease, and other compounds deposited on the fiber. In terms of numbers of sites occupied by the fungicide, a mold spore would appear rather like a tennis ball placed on a fine hair brush, *i.e.,* there would be sufficient toxic groups present to attack the spore. The range of the fungicidal action would, however, be limited by the length of the molecule of the compound in question, and quite a thin film of grease or other material on the fiber would very effectively isolate the spore from the fungicidal group. The greater the length of the chain attaching the fungicidal group to the substrate, the greater the chance of the barrier being breached. This was the basis of the approach to the problem made by Picklesimer (2), using tetrakis(hydroxymethyl)phosphonium chloride.

In view of these considerations, it was realized at quite an early stage in this investigation that it might be necessary to look for a compound which could be bound to collagen and which also possessed a fungicidal group that could be split off, preferably under conditions favoring mold growth. In this way fungicide would be continually replaced as it was leached out from the leather in wear. In the event, this proved to be the most useful approach.

Consideration of the reaction of collagen with various compounds indicates that the amino groups of the lysine residues are the sites most likely to be useful for the binding of potentially fungicidal compounds to collagen. Not only is there a wide variety of compounds that will react with these groups, but reaction

generally takes place under relatively mild conditions. Unfortunately, the number of amino groups in collagen is rather low, namely, 30–34 moles per 10^5 g., and only about 20–25 of these appear to be readily available for reaction.

Binding of a fungicidal compound can occur either directly with the amino groups or by means of a bi- or polyfunctional intermediary. Examples of compounds that can be bound directly are aldehydes, isocyanates, acid chlorides, and acid anhydrides. As an intermediary, cyanuric chloride offers interesting possibilities. Because of the differing ease with which successive chlorine atoms in the triazine ring can be replaced, it is possible to prepare mono- and disubstituted derivatives of compounds containing hydroxyl, amino, and thiol groups. These can then be readily incorporated into skin by reaction of the remaining chlorine atoms or atom with the amino groups of lysine residues. This is the principle of the Procion and Cibacron ranges of dyestuffs. Results obtained with cyanuric chloride and dichlorotriazines suggest that about one third of the amino groups react in this way at pH 8 to 9.

EXPERIMENTAL

Raw Material

Acetone-dehydrated skin prepared from commercially limed and pickled hair sheepskins and standard hide powder (Batch No. C24) were used in all tests.

Preparation of Dichlorotriazine Derivatives

Cyanuric chloride, the starting material in all the preparations, was purified by dissolving the laboratory grade material in the minimum volume of carbon tetrachloride, filtering, and evaporating to dryness.

p-Nitrophenol (PNP) (3), 2-mercaptobenzothiazole (MBT), and *o*-chloroaniline (OCA) (4) were reacted in molar proportions with cyanuric chloride (CC) dissolved in acetone or in the form of a fine precipitate formed on the addition of the acetone solution to ice-cold water. In order to prevent formation of the disubstituted monochlorotriazine derivatives, the reaction mixtures were cooled in ice and reagents added at a controlled rate to prevent overheating. Since the reaction involves formation of HCl, it was assisted by the addition of a base. Those used were collidine (PNP), sodium hydroxide (MBT), and sodium carbonate (OCA). Times of reaction were up to one hour, after which any water in the system was removed at as low a temperature as possible with the minimum of delay. All three compounds prepared were recrystallized from benzene.

The melting points of the derivatives were: PNP-CC 198–200°C.; MBT-CC 155–6°C.; OCA-CC 158°C. Infrared spectroscopic examination of the compounds indicated that none contained more than one percent of the original, unreacted fungicide.

Mold Resistance Tests

Pure cultures of the following organisms were kindly supplied by the Woodstock of Agricultural Research Centre of Shell Research Ltd.:

1) *Aspergillus niger*
2) *Chaetomium globosum*
3) *Penicillium roqueforti.*

The first was chosen because of its common occurrence in nature and affinity for protein materials; the second is widely used in tests on cellulosic materials; and the third is another commonly occurring mold. The organisms were recultured on potato dextrose agar every few weeks.

In the preliminary screening tests, small samples of the treated skin were sterilized by exposure to propylene oxide vapor, wetted back in sterile water, and then pressed firmly on to the surface of potato dextrose agar gel (Oxoid C M139) substrate in a petri dish. Mold spores from a pure culture were transferred to the gel by a sterile wire loop, inoculation being made either in a circle around the sample or on to the sample itself.

Toxicity of cyanuric chloride derivatives was tested by dispersion, using a little wetting agent (Teepol L), and incorporation into an agar gel, which was then inoculated with an aqueous suspension of *Aspergillus niger.*

Leathers to which the fungicides had been bound by means of cyanuric chloride were tested by the mycelial mat procedure of Dahl and Kaplan (5).

Determination of Fungicides

PNP, MBT, and OCA were determined spectrophotometrically in chloroform at their respective absorption maxima, 310, 330, and 290 mμ.

PNP in aqueous solution was determined by measurement of absorption at the isosbestic point, 347 mμ, to avoid complications due to variation of absorption with pH. A colorimetric procedure based on the formation of an indophenol blue color measured at 630 mμ was also used (Lollar (6)).

Spectrophotometric measurements at fixed wavelengths were made in one-cm. cells using a Unicam SP500. Wavelength scans were made with a Unicam SP800 recording spectrophotometer.

RESULTS

Preliminary Screening Tests

In the first instance, acetone-dehydrated calf or sheepskin was reacted directly with a number of compounds considered likely to confer fungicidal resistance, and also with triazine derivatives of a number of recognized fungicides. After treatment, the samples were washed well in water and acetone-dehydrated. In some instances they were also Soxhlet extracted with diethyl ether to remove unreacted

TABLE I

TESTS FOR FUNGICIDAL ACTIVITY

Treatment	Amino Groups Substituted (Moles per 10^5 g.)	*Aspergillus niger*		*Penicillium roqueforti*		*Chaetomium globosum*	
		Not Extracted	Extracted	Not Extracted	Extracted	Not Extracted	Extracted
Cyanuric chloride and derivatives							
Cyanuric chloride	12	++	○	+	+	++	++
o-chloroaniline	13	+++	+	—	—	+	+
2-mercaptobenzothiazole:							
pH 8	12	+++(Z)	+++(Z)	++	++(Z)	++(Z)	++(Z)
pH 9	9	+++(Z)	+++(Z)	+++(Z)	+++(Z)	++	++
p-nitrophenol	12	++(Z)	+	++	++(Z)	++	+++
Two-stage — Cyanuric chloride followed by:							
o-chloroaniline	20	+++(Z)	○	+	+	++(Z)	○
2-mercaptobenzothiazole	19	+++(Z)	+++(Z)	++	++	++	+++
p-nitrophenol	5	+++(Z)	+++(Z)	+	+	++	++
Formaldehyde and Formaldehyde Generators							
Formaldehyde	—	+		++			
Dowicide Q	15	+		○			
Monomethyloldimethyl urea	14	+		++			
Miscellaneous Compounds							
Trivinyl sulfone (Sulfix A)	27	+++(Z)		+++		++	

○ = no activity; + = slight activity; ++ = moderate activity; +++ = good activity; (Z) = zone of inhibition around sample

derivatives. Small samples of the treated pieces were tested by placing on potato dextrose agar gels and inoculation with spores of *Aspergillus niger, Chaetomium globosum,* and *Penicillium roqueforti.*

Direct treatment with acrolein, acrylonitrile, phenyl isocyanate, chloroacetyl chloride, benzenesulfonyl chloride, and S-acetyl mercaptosuccinic anhydride conferred little or no fungicidal properties, nor did treatment with the methoxy, thioglycollate, and hydrosulfide derivatives of cyanuric chloride.

Some results with the more effective compounds are recorded in Table I. The most effective of the reactive compounds was the 2-mercaptobenzothiazole derivative of cyanuric chloride, followed by the *p*-nitrophenol and *o*-chloroaniline derivatives and Sulfix A (trivinyl sulfone). Solvent extraction to remove uncombined material had little or no effect on activity, except with the *o*-chloroaniline derivative. Since the formol titration indicates quite extensive reaction, the marked loss of activity is surprising; it perhaps indicates that, on being bound to the protein, the *o*-chloroaniline derivative can no longer exert its fungicidal action.

The more promising compounds were tested further by inoculation of *Aspergillus niger* directly on to the treated skin samples. After three days' incubation at 30°C. there was considerable mold growth on all of them, whereas samples of skin impregnated with the traditional fungicides (*p*-nitrophenol, pentachlorophenate, or cresylic acid) showed little or no growth. The results from direct inoculation were rather erratic, and this may partially account for these rather disappointing findings.

Further consideration was given to alternative methods of testing, and the possibility of using a procedure based on oxygen consumption of the mold (7–9) was briefly considered. Such methods have the advantage of giving a quantitative assessment of toxicity, and to this extent are to be preferred to the more subjective tests. For the present purpose, however, it was considered that direct observation of mold growth on the treated samples would be the most useful guide to potential fungicidal properties. The procedure finally adopted was the mycelial mat technique described by Dahl and Kaplan (5), using a potato dextrose agar medium.

Triazine Derivatives as Fungicides

The preliminary tests showed that the cyanuric chloride derivatives of *p*-nitrophenol (PNP), 2-mercaptobenzothiazole (MBT), and *o*-chloroaniline (OCA) conferred some fungicidal resistance when bound to skin. These compounds were, therefore, studied further.

Tests involving incorporation into agar gels and inoculation with *Aspergillus niger* indicated that the derivatives were only about one fifth as toxic as the parent compounds, but nevertheless still showed appreciable activity at 0.1 percent concentration.

Sheepskin pieces were treated with the derivatives in 50 percent aqueous ace-

tone at pH 8 to 9, and then lightly vegetable-tanned and fatliquored. The treatment was carried out before tanning, so that an estimate of the degree of interaction could be obtained by the formol titration (10). Using this procedure, about ten moles per 10^5 g. or one third of the amino groups were found to have reacted with both derivatives. The shrinkage temperature was increased (PNP-CC 68°C.; MBT-CC 60°C.), indicating some degree of crosslinking, especially with the PNP derivative. Even if all the amino groups substituted were involved in crosslinking, so that only five moles of derivative were bound, there would still be about 0.7 percent PNP and 0.9 percent MBT incorporated, sufficient to give complete protection. Comparable samples were also treated with the free compounds dissolved in acetone or water, and these, together with the experimental samples, were either tanned directly or first extracted with acetone and water.

The results of tests using the mycelial mat technique of Dahl and Kaplan (5) are summarized in Table II. With PNP all leathers were free of mold; pre-

TABLE II

MOLD GROWTH ON TREATED VEGETABLE-TANNED LEATHER

(*Aspergillus niger*)

		Extracted With	
	Not Extracted	Water	Acetone
Control — No fungicide	++	++	+++
p-Nitrophenol			
Applied in water	○	+	+++
Applied in acetone	○	+++	+
Triazine derivative	○	○	○
Mercaptobenzothiazole			
Applied in acetone	++	++	++
Triazine derivative	+	++	++

○ = surface of leather completely free from mold growth.
+++ = moderate mold growth.

sumably, sufficient of the free material incorporated before tanning still remained to give complete protection. MBT was apparently much less effective. Extraction with water or acetone before tanning reduced the resistance of the leathers treated with the free compounds. The PNP-derivative still gave complete protection, but there was no evidence that the leather containing the combined MBT derivative had any greater resistance than the controls. It is perhaps worth noting that PNP, when applied in water, appears to have some substantivity to water and, when applied in acetone, some substantivity to acetone.

On the basis of these results, it seemed that with the PNP derivative some degree of fungitoxicity was retained, whereas with the MBT derivative this was doubtful. However, chloroform-extraction of the leather treated with PNP-CC revealed the presence of about 0.1 percent free PNP, sufficient to account for its resistance to mold attack. Since the treated skin contained only 0.04 percent PNP before tanning, there was presumably breakdown during tanning and/or in the mold test. It seemed, therefore, that the PNP-CC and other triazine derivatives might fulfill the requirements outlined earlier for a compound which can be covalently bound to collagen and which releases a fungitoxic group under conditions conducive to mold growth.

Breakdown of PNP-CC in aqueous suspension and when bound to collagen was examined. In aqueous suspension at 30°C. it was possible to follow this by changes in the ultraviolet absorption, measurements being made at the isosbestic point (347 mμ) to avoid complications due to variations in pH. At pH 13.0 breakdown was rapid, approaching completion in two days; at pH 7.0 it was very much slower, averaging about 0.1 percent per day initially, decreasing to less than 0.01 percent per day over longer periods; and at pH 1.0 it increased again (Table III).

TABLE III

BREAKDOWN OF *p*-NITROPHENOL DERIVATIVE OF CYANURIC CHLORIDE IN AQUEOUS SUSPENSION* AT 30°C.

	Time of Storage (Days)							
pH	1	2	5	7	15	23	58	93
	Free PNP Produced — mg.							
1	1.03	1.93	3.74	5.36	10.08	13.80	25.3	32.1
7	0.19	0.25	0.35	0.42	0.57	0.74	0.91	0.92
13	36.4	40.9	45.8	45.8	47.1	47.6	48.2	48.5

*(100 mg. PNP-CC [estimated PNP equivalent = 48.2 mg.] in 100 ml. 0.1 N HCl [pH 1], phosphate buffer [pH 7] or 0.1 N NaOH [pH 13].)

Hide powder treated with the derivative was adjusted to pH 4.0 and 8.0 and stored at 50 or 100 percent relative humidity and 30°C. Immediately before storage, the hide powder was extracted with chloroform in a cold Soxhlet to ensure that no free PNP was present. The total PNP available as the derivative at the start of the experiment was determined by hydrolysis, extraction of the hydrolysate with chloroform, and determination of PNP in the extract by U.V. absorption measurements. A correction for losses during hydrolysis was made, based on recovery of PNP added to hide powder, and hydrolyzed under corresponding conditions.

Two series of tests were carried out, one in which the treated hide powder was extracted after one month and again after two and five months to remove PNP

released (A), and a second in which the PNP was allowed to accumulate throughout the full time of storage, only small samples being taken for determination of the PNP released at different times (B). The free PNP was determined by extraction with chloroform in a cold Soxhlet, followed by reduction to *p*-aminophenol and coupling with phenol (6). This more specific colorimetric procedure was preferred to U.V. absorption measurements because of the interference from protein degradation products, especially after the longer periods of storage.

Results of the two series are summarized in Figure 1. The general inference is that breakdown occurs rather more rapidly at pH 8.0 than at pH 4.0, and is almost twice as rapid at 100 percent relative humidity as at 50 percent relative humidity. If the PNP is allowed to accumulate (Series B — dotted lines), breakdown of the derivative is retarded and appears to be reaching a limiting value of about 0.1 percent on the protein (20 percent breakdown of PNP-CC) at 50 percent relative humidity and between 0.15 and 0.2 percent (30–40 percent breakdown) at 100 percent relative humidity, depending on the pH. This retardation is also apparent to some extent in the A series, the rate of breakdown being appreciably less when the interval between extractions was three months as compared with one month.

In practical terms, the results indicate that breakdown will be relatively slow under normal conditions of storage, and will fall off to almost negligible proportions as the PNP accumulates. Under conditions conducive to mold growth breakdown will be more rapid, and any PNP leached out in wear will be replaced relatively quickly.

Other fungicides capable of reacting with cyanuric chloride may give derivatives of differing stabilities. The behavior of compounds bound to the triazine residue through mercapto and amino groups was, therefore, examined. The derivatives were reacted with hide powder at pH 8.0 under the same conditions as those used for PNP-CC, and excess derivative and any free compound removed by extraction with acetone and chloroform.

From the formol titration it was estimated that between two and five moles amino groups per 10^5 g. were substituted by MBT-CC, and four to eight moles by the OCA derivative. Direct determination of the amounts bound by hydrolysis and extraction was less successful than with PNP-CC. With the MBT-CC treated collagen, the chloroform extract of the hydrolysate gave a U.V. spectrum typical of free MBT, with a peak at 330 mμ and with no indication of the presence of the derivative. Recovery of MBT added to hide powder, however, was only 25 percent. Applying a correction factor based on this recovery, a value of 4.8 moles MBT per 10^5 g. or 0.8 percent was obtained. With the OCA derivative the chloroform extract of the hydrolysate gave a confused picture; it resembled that of neither OCA nor OCA-CC, but could have resulted from a mixture of the two. The inference is that the N-C bond is only partially cleaved dur-

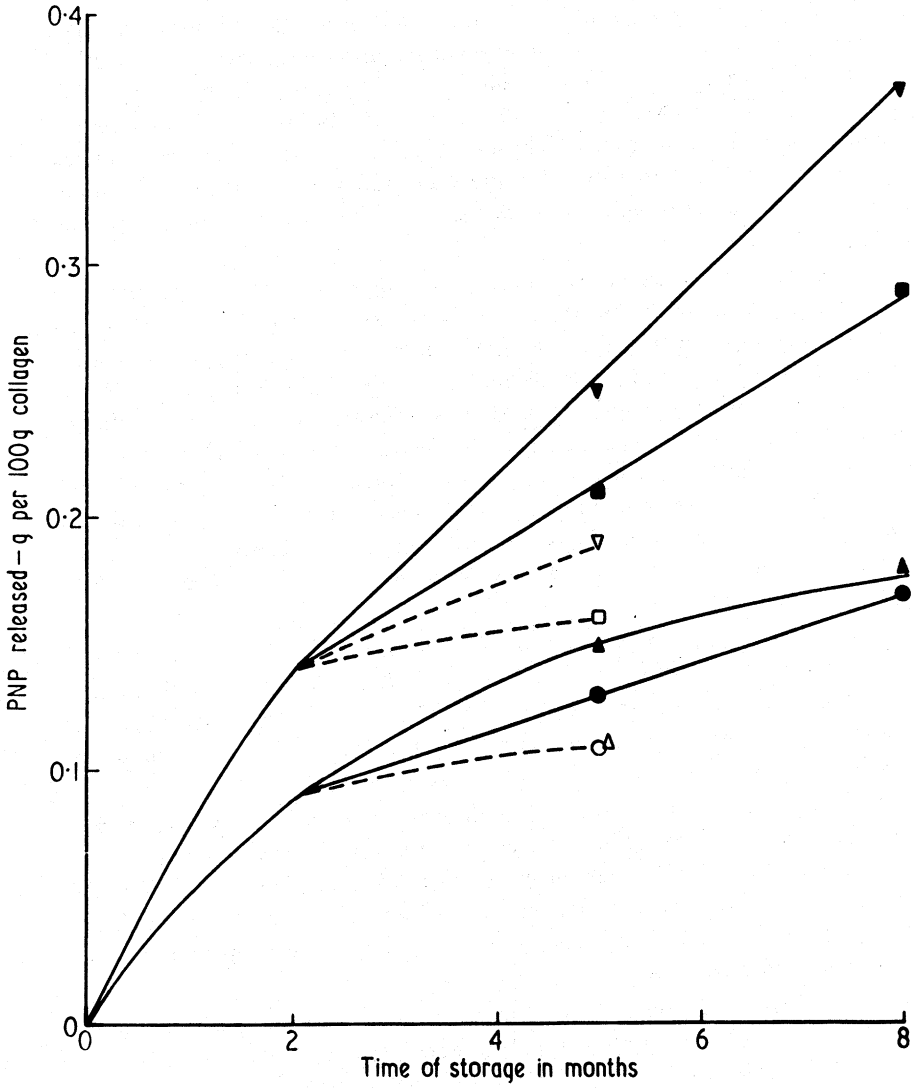

FIGURE 1.—Breakdown of *p*-nitrophenoxy-dichlorotriazine bound to collagen during storage at 30°C. Total PNP bound to collagen: 0.55 g. per 100 g. collagen.

○ equilibrated at pH 4.0 — stored at 50% r.h.
△ equilibrated at pH 8.0 — stored at 50% r.h.
□ equilibrated at pH 4.0 — stored at 100% r.h.
▽ equilibrated at pH 8.0 — stored at 100% r.h.

Series A — free PNP removed at intervals — solid symbols.
Series B — free PNP allowed to accumulate — open symbols and dotted lines.
At one and two months, curves for the two series at both pH values were coincident.

ing hydrolysis, and is hence more stable than either the O-C or S-C bonds of the other two derivatives.

The low solubility of both the MBT and OCA derivatives made it impossible to follow their breakdown in aqueous suspension by any simple means, and breakdown tests were confined to storage of the treated hide powder at pH 8.0 and 30°C. With the MBT-CC treated hide powder, the chloroform extracts gave a well defined U.V. spectrum characteristic of free MBT, and measurements of the peak at 330 mμ gave values for free MBT of 0.03 and 0.06 percent on dry protein weight after storage for three months at 50 and 100 percent relative humidity, respectively. Assuming an initial available MBT content of 0.8 percent, this corresponds to 2.4 and 4.8 percent breakdown, compared with 20 and 40 percent for the PNP derivative under corresponding conditions. The extract from the OCA-CC treated hide powder showed no increased U.V. absorption, indicating that breakdown was negligible.

Limited work was also carried out on the binding of the PNP derivative to wool and cotton. With the former, about 0.7 percent on dry weight was bound, indicating that both amino and thiol groups are involved. With cotton, binding was less successful since, under conditions necessary for reaction with the hydroxyl groups of cotton, extensive breakdown of the derivative itself occurred.

DISCUSSION

A recurring question in this work has been whether any fungicidal activity is retained by a covalently bound compound. A study of the literature indicated that this might be possible as far as the substrate to which it is bound is concerned, but that its reduced mobility must decrease its effectiveness and preclude the extension of its activity to other substrates.

It was found that direct binding of a number of potentially fungitoxic compounds to skin conferred relatively poor resistance to mold growth compared with the incorporation of free fungicides, for example, *p*-nitrophenol and pentachlorophenate; also, that the fungitoxicity of the triazine derivatives of *p*-nitrophenol and mercaptobenzothiazole is appreciably less than that of the free compounds. The chances of a bound fungicide's being effective as far as leather was concerned, therefore, seemed relatively slight, and it was considered that a more useful approach to the attainment of more permanent fungicidal resistance would be through the binding of a compound from which a fungitoxic group would be slowly released, preferably only under warm moist conditions, when the risk of mold growth is greatest.

It has been shown that *p*-nitrophenol, an effective leather fungicide, can be bound covalently to skin, using cyanuric chloride as an intermediary. The derivative so bound breaks down slowly under normal conditions of storage to give free PNP, and more rapidly under warm moist conditions. In this way PNP leached out from the leather during wear can be replaced when it is most needed

for the inhibition of mold growth. A study of the rate of breakdown indicates that under normal conditions this is slow, and is unlikely to lead to the accumulation of more than about 0.1 percent PNP in the leather, while under conditions conducive to mold growth breakdown will be more rapid and replacement should be at a sufficiently rapid rate to ensure adequate protection. Protection over periods from one to three years may reasonably be expected, depending on the climatic conditions and on the rate at which PNP is leached out.

Other fungicides capable of reacting with cyanuric chloride can presumably be used similarly, and by varying the nature of the bond between the fungicide and the triazine residue it is possible to vary the rate at which the free fungicide is liberated. For example, mercaptobenzothiazole, which is bound through its thiol group, is released more slowly than *p*-nitrophenol. It may, therefore, be more suitable than the PNP derivative in some circumstances, for example, when prolonged storage before wear is envisaged, or under conditions where leaching of the fungicide is likely to be slow and the necessity for rapid replacement is less. When binding of the fungicide is through an amino group, the derivative appears to be stable, and there is little or no liberation of the free fungicide even at high humidities.

In these studies, the triazine derivative was bound to the collagen before tanning. In practice it would be necessary to carry out the treatment after tanning, and the relatively high pH required for effective reaction, *i.e.*, pH 8.0, presents some problems. The fact that appreciable binding of Procion dyes by chrome leather is possible suggests that such difficulties can be overcome.

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